Diagnostic Advances for Improved Treatment of Childhood Acute Lymphoblastic Leukemia in Indonesia

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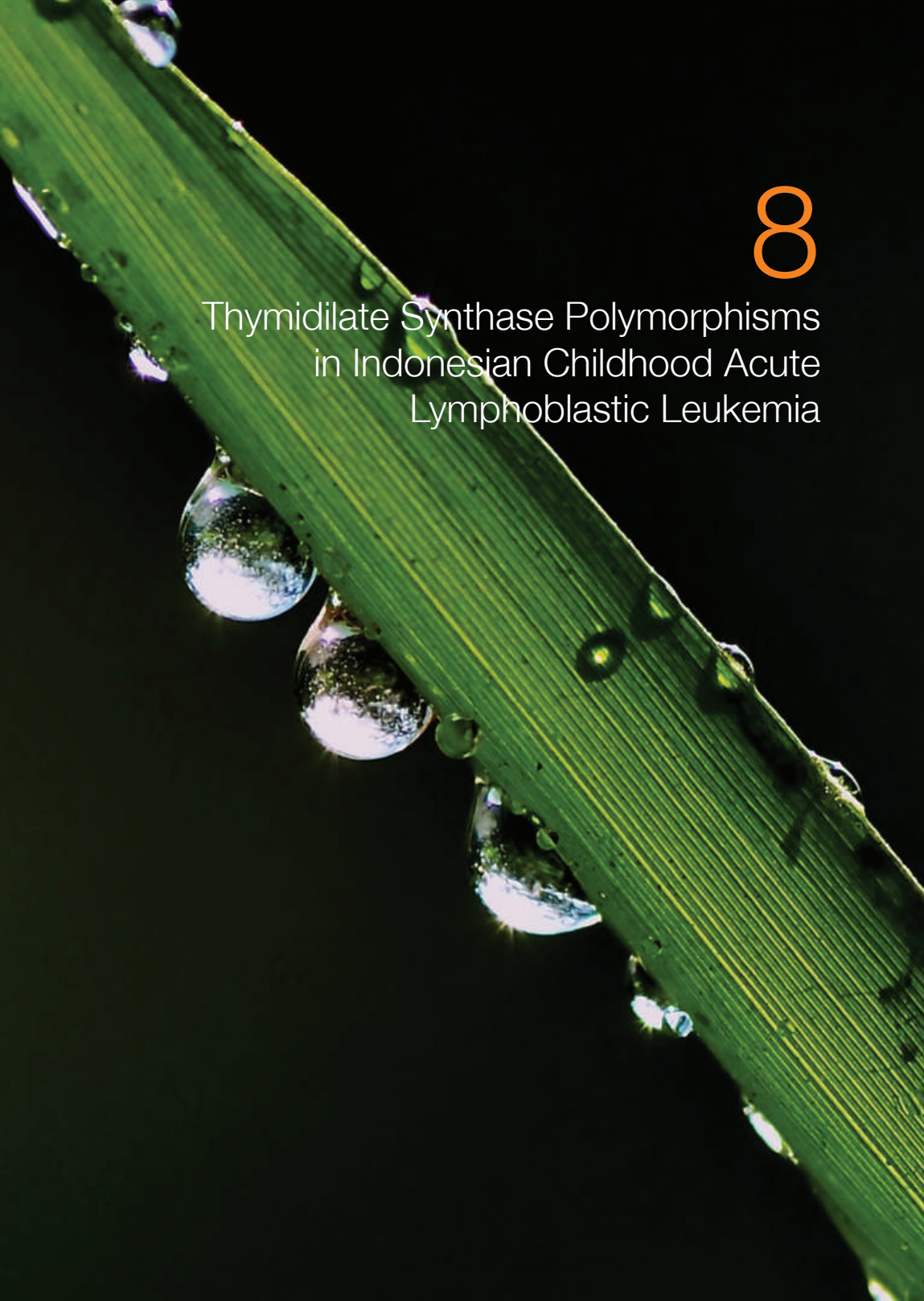

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Thymidilate Synthase Polymorphisms in Indonesian Childhood Acute Lymphoblastic Leukemia

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ABSTRACT

The enzyme thymidylate synthase (TS) converts dUMP into dTMP and requires folates as co-factors. Since dTMP is necessary for DNA synthesis, TS is an important target of cancer chemotherapy. Ethnic variations of the polymorphic tandem repeat sequence in the enhancer region of the TS promoter, has previously been described to influence the outcome of acute lymphoblastic leukaemia (ALL). A triple repeat is associated with a higher *TS* gene expression than a double repeat, resulting in poorer outcome of ALL patients treated with the anti-folate methotrexate (MTX). In addition, the association of *TS* expression and MTX resistance appears to be even more pronounced in combination with an accumulation of folates. This latter event can occur when methylenetetrahydrofolate reductase (MTHFR) has impaired enzyme activity due to a *C667T* mutation. In this study, we determined the ethnic variations of the *TS* and *MTHFR* genotype between Caucasian an Indonesian in ALL cells obtained at diagnosis from 157 Caucasian and 101 Indonesian children with MTX-treatment in prospective. Furthermore, we determined the involvement of *TS* polymorphisms in MTX sensitivity, by using a *TS* inhibition assay (TSIA). Homozygous *TS* triple repeats were more than twice as common in Indonesian samples (76.3%) than in Caucasian samples (33.1%). Heterozygotes of the *MTHFR* mutations were seen in 15% of the screened Indonesian samples. These results demonstrate significant ethnic variation in a *TS* gene regulatory element in leukemic cells. A difference was found between the MTX sensitivity and the presence of a double or triple repeat in the Caucasian ALL group. Interestingly, according to these data it seems that the samples with a triple repeat show a shift in their distribution towards hypersensitivity to MTX, in contrast to previously described results. However four of the samples (16.0%) did show resistance to MTX, which may suggest an additional role for other enzymes, such as *MTHFR*. Further investigation in the Indonesian samples may give insight in the role of the polymorphisms in MTX sensitivity.

Keywords: thymidylate synthase, acute lymphoblastic leukaemia, polymorphism, ethnic variations, childhood, Indonesia.

Introduction

A major problem in the treatment of acute lymphoblastic leukemia (ALL) is resistance to the chemotherapeutic agents. An important chemotherapeutic agent used in the treatment of ALL is the anti-folate methotrexate (MTX), which inhibits DNA synthesis. The polyglutamylated form of MTX is an anti-folate and acts as an inhibitor of the enzyme thymidylate synthase (TS) (Rots MG et al., 2000), which is an important enzyme involved in the folate-metabolism. There are several underlying mechanisms responsible for resistance to MTX. One of the mechanisms is intrinsic resistance by genetic changes in enzymes involved in the folate-metabolism. Modulation of TS, or of other folate-enzymes such as the *methylenehydrofolate reductase (MTHFR)*, may result in drugs resistance to anti-folates.

The first important enzyme TS whose gene is located on chromosome 18p11.32, catalyses the conversion of deoxyuridylate monophosphate *(dUMP)* to deoxythymidylate monophosphate (dTMP) (fig. 1). TS is a key-enzyme in de novo DNA synthesis. Inhibition of this enzyme results in deoxythymidine triphosphate depletion and thereby chromosome breaks and cell death (Welsh SJ et al., 2000). Impairments of the *TS* enzyme have been associated with chromosome damage and fragile site induction (Hori T et al., 1984, 1985). TS has aunique tandem repeat sequence inthe 5′untranslated region (UTR) immediately upstream of the ATG codon initiation start site that has been shown to be polymorphic, containing either two or three 28-bp repeats (Fig. 2) (Hori T et al., 1985). In colorectal cancer, the presence of the triple versus double 28-bp repeat was shown to enhance gene expression in in vitro and in vivo studies (Horie N, 1995; Kawakami K, et al., 1999; Pullarkat ST et al., 2001) and associated with poor prognosis (Johnston PG et al., 1992).

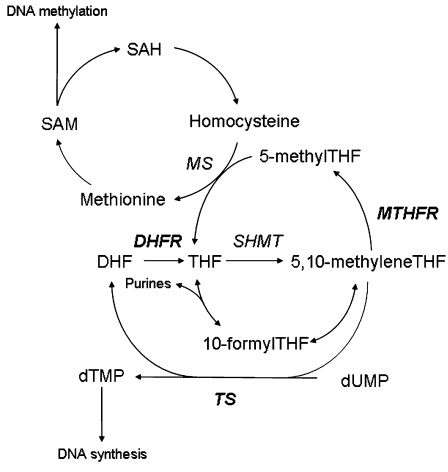

Figure 1. Overview of the human folate metabolic pathway

Marsh et al, 1999, have first described ethnic variation between different populations in the TS enhancer region (Marsh, 2004; Marsh et al., 1999; Marsh S et al., 2000). In childhood ALL homozygosity for the TS triple repeat, was reported to be associated with poorer outcome than in those with at least one double-repeat allele (Krajinovic M et al., 2002). However, in adult ALL, polymorphisms of the *TS* triple repeat seem to result in a higher level of protection in ALL risk than a double repeat (Hishida A et al., 2003; Skibola CF et al., 2002).

Thymidilate synthase (TS) binds methylenetetrahydrofolate (methyleneTHF), which serves as a hydroxymethyl donor in the conversion of dUMP to dTMP in the DNA synthesis pathway. In addition to polymorphisms of the repeat region, also other mutations have been described to play a role in MTX sensitivity. The second important enzyme involved in MTX sensitivity is *MTHFR* that plays a role in the intracellular folate homeostasis by catalysing the irreversible conversion of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF) (Frosst P et al., 1995; Scott J and Weir D, 1994). The substrate 5,10-methyleneTHF is required for DNA synthesis and for maintaining the balance of the nucleotide pool, whereas 5-methylTHF is required for methylation reactions (Frosst P et al., 1995; Goyette P et al., 1994). *MTHFR* gene is located on chromosome 1p, and is subject to several polymorphisms (Rozen R, 1996). Among them a mutation C→T at nucleotide position 677 (exon 4), which results in an alanine to valine substitution (Frosst P et al., 1995; Goyette P et al., 1994). This mutation is associated with lower enzyme activity and increases frequency of the mutated *677*

TT genotype is around 10-15% in Caucasian, termolability of *MTHFR*, which leads to lower levels of 5-methylTHF, an accumulation of 5,10-methyleneTHF, and increases in homocysteine levels. Theand is related to increased risk of congenital neural tube defect and to colorectal cancer (Ueland PM et al., 2001).

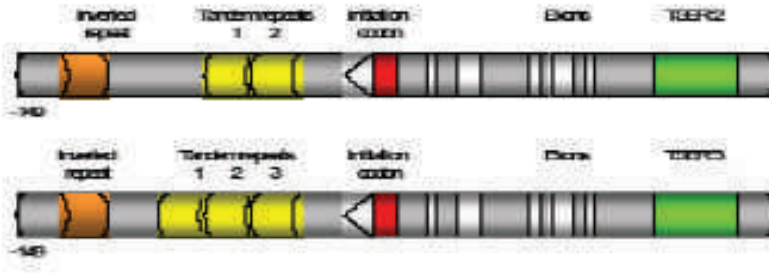

Figure 2. Schematic structure of TS Enhancer Region, containing either double or Triple 28-bp repeat in the 5'untranslated region (UTR).

An accumulation of 5,10-methyleneTHF resulting from the *MTHFR C677T* polymorphism (Bagley PJ and Selhub J, 1998) may also have an effect on the response of cancer cells to the chemotherapeutic agent MTX because the activity of the drugs is dependent on a competitive interaction with folate metabolism. 5,10-methyleneTHF is a substrate for the *TS* enzyme. Because of the prevalence of the TS tandem repeat and *MTHFR C677T* polymorphisms and the fact that MTX is a common chemotherapeutic agent used for the treatment of pediatric ALL, identification of these polymorphisms may be an important pharmacogenetic determinant of predicting response to MTX. To our knowledge, identification of the *TS* and *MTHFR* polymorphisms in Indonesian ALL patients has not been performed before. We also compare the ethnic variations in *TS* and *MTHFR* between Indonesian and Caucasian ALL populations and the involvement of these different polymorphisms in MTX sensitivity.

Materials And Methods

Study population

Identification of polymorphisms in leukemic cells was performed on individuals with different ethnic background diagnosed with ALL. Samples were obtained from cells frozen in N2 (l) vials, fresh bone marrow or peripheral blood, bone marrow slides or cytospins.

Indonesian population

The patients included in this study were children diagnosed with ALL treated at Dr. Sardjito Hospital, Yogyakarta and Dr. Soetomo Hospital, Surabaya with the Wijaya Kusuma ALL 2000 protocol. Vials and bone marrow samples were available from 101 patients. The population consisted of 42 female and 53 male patients and from 6 patients these data were missing. Ages ranged from just after birth till 14 years old. The ALL subtypes were morphological classified according to the French-American-Britisch (FAB) criteria. From the 101 Indonesian patient samples, 79 had FAB type L1; 11 had L2: 2 had L3 and from 9 samples FAB type was not available.

Caucasian population

The study population included 157 childhood ALL of the Netherlands, Germany, United Kingdom and France. Patients of Caucasian origin were treated according to different protocols. The patients population consisted of 57 female, 82 male, and 18 unknown ranging in age from just after birth till 16 years.

Isolation of mononuclear cells from fresh bonemarrow blood

Full blood was diluted 1:1 with wash medium (Phosphate Buffered Saline, PBS pH 7,4 and 1% Fetal Calf Serum, FCS) and put on a ficoll-gradient with ratio 2:1. The interphase was collected and the cells were washed two times with wash medium. The mononuclear cells were dissolved in 500 µl wash medium. Cell count ranged from 10.0-16.7 x 10^6 total cells.

Thawing of nitrogen stored vials

The vials stored in the nitrogen tank were thawed in a 37°C water bath, transferred in a tube and diluted in 5 ml PBS + 0.1% Bovine Serum Albumin (BSA). After centrifugation and washing with wash medium the pellet was suspended in 200 µl PBS + 0.1% BSA.

DNA isolation

Genomic DNA from the 101 patient samples was extracted. The mononuclear cells that were available came from vials and bone marrow slides therefore DNA isolation was performed in two different ways. 1) DNA extracted from cells stored in liquid nitrogen was obtained using Qiagen DNA Tissue Kit (Qiagen, Hilden, Germany). The DNA isolation was preformed according the protocol included in the Kit. Because

of using cells instead of tissue the incubation step with proteinase K was reduced from overnight to one hour. The DNA was finally suspended in 20 µl double distilled water (DDH) and stored at -20°C. 2) Before DNA was isolated from bone marrow slides an extra step with HBSS (Hank's Balanced Salt Solution) and shock buffer was used for a better separation and purification between the mononuclear cells and erythrocytes. An overnight treatment was followed by phenolchlorofrom-isoamylalcohol (FCI,25:24:1) extraction. DNA was precipitated with 3M Sodium Acetate (1/10 of total volume) and 2.5 volumes of 100% ethanol. Glycogen (4g) was used because we expected a small amount of DNA. This pellet was suspended in 20 µl DDH and stored at -20°C.

Genotyping/gene polymorphisms

TS and *MTHFR* genetic polymorphisms were analyzed by Genescan and Lightcycler, respectively. All isolated DNA samples from Indonesia (n=101) were screened on TS polymorphism while 15 samples were screened on *MTHFR* polymorphisms. Data of the polymorphisms and the MTX sensitivity from the Caucasian patients was already available from previous experiments.

Determination of TS polymorphisms

For *TS* polymorphisms, forward en reverse primers were 5'- GCT CCG AGC CGG CCA CAG GCA TGG CGC GG-3' and 5'- GTG GCT CCT GCG TTT CCC CC-3' respectively. Eurogentec synthesized primers used for PCR products visualized on gel. Primers used for gene scan needed to be labeled with a fluorescein for measurement. The reverse primer was labeled with FAM and synthesized by Isogen Bioscience BV, Maarssen, the Netherlands. Presence of the tandem repeat sequence in the 5'-terminal of the regulatory region of the TS gene, a fragment containing the 28-bp repeats, was amplified and detected using a protocol described by Horie et al, 1995 and Marsh et al, 1999. PCR were run on a Peltier Thermal Cycler - 20° in a 50 µl final volume containing 100 ng of genomic DNA, 1 mM $MgCl_2$, 5.0 µl of Mg-free buffer 10x (Amersham Biosciences), 0.2 mM dNTPs, 0.2 µM of each primer, 10% DMSO and 2.5 units of Taq DNA Polymerase (5U/µl; Amersham Biosciences). The reactants were added and mixed on ice and transferred to a preheated 94°C block for amplification of 35 cycles. Each cycle of amplification consisted of denaturation for 1 min. At 94°C, annealing for 1 min. At 60°C and extension for 2 min. at 72°C followed by 5 min. at 72°C after the last cycle. The amplified DNA fragments were visualized on a 2% agarose gel (RESponseTM Research PCR agarose, 335010 BIOzym) with ethidium bromide. Homozygotes for the double repeat *(2R/2R)* produced a singlet 210-bp band. Heterozygotes *(2R/3R)* produced 210-bp and 238-

bp fragments and homozygotes for the triple repeat *(3R/3R)* produced a 238-bp fragment. As a positive control a heterozygous cell line LS 174T was used. Parts of the PCR products were visualized using gel electrophoresis and checked with the Genescan (ABI prism® 3100 - Avant, Applied Biosystems). Since the latter method was more sensitive all further samples were only visualized on the Genescan. The genomic PCR assay was performed again on all the isolated Indonesian samples, except that the reverse primer was labeled at the 5'-end with fluorescein-FAM (Isogen Bioscience BV). PCR products (5 µl) were combined with 12,5 µl loading mixture consisted of 12 µl Hi-Di formamide and 0.5 µl Gene ScanTM -500 ROX TM Size standard (Applied Biosystems, Foster City, CA). The samples were run on the ABI PRISM sequencer (3100 genetic Analyzer; Applied Biosystems, Foster City, CA) and analyzed using Genescan Analysis software (version 1.2, Applied Biosystems).

Determination of *MTHFR* polymorphisms

For the identification of single nucleotide polymorphisms in the *MTHFR* gene the primer pair, the fluorescent detection probe and anchor probe for determination were provided by Genes-4U (*MTHFR C677T* Tool Set TM for Light Cycler TM). Determination of *MTHFR* polymorphisms, The *C677T* (Ala→Val) mutations were analyzed by means of melting curve analyses on Lightcycler (Roche Diagnostics), using the *MTHFR C677T* Tool Set TM for Light Cycler TM (Genes 4U, licensed by Roche Diagnostics GmbH) based on the protocol recommended by the manufacturers. The 3' end of one probe was labeled with the fluorescent donor dye Fluorescein (FLU), whereas the 5' end of an adjacent probe was labeled with an acceptor dye, LC-Red640 as the anchor probe. After reaching the annealing temperature the hybridization probes are allowed to bind to their specific target region. By increasing the temperature the probes melt away at their own specific melting temperature and at that moment the donor dye comes into close proximity to the acceptor dye and Fluorescence resonance energy transfer (FRET) occurs which results in a fluorescence signal. A sensor probe with a mismatch (containing the mutation) will melt at a lower temperature than a perfectly matched probe (containing the wild type gene). The lightcycler PCR amplification was run in 20 αl final volume. Reaction mix (16 µl) containing 4 αl Faststart DNA Master Hybridization Probes plus 5x (LC FastStart DNA masterplus hybridisation probes, 0351567 Roche Diagnostic, France) 2.8 αl Oligo Tool MTHFR 677, 9.2 αl solvent MTHFR 677 (Genes 4U, licensed by Roche Diagnostics GmbH) and 4 αl genomic DNA (3 ng). After DNA denaturation and enzyme activation (10 minutes at 95°C), DNA was amplified for 50 cycles (1 s at 95°C, 15 s at 57°C and 5 s at 72°C). After amplification, melting curves of the DNA-probes complexes synthesized were measured by increasing temperature gradually (0.10C/s) up to 95°C. The mutated form of *MTHFR C677T* is visualized based on the

fact that the melting temperature of the DNA/probe complex is lower in the case of DNA-probe T/C mismatch at nucleotide 677 compared with the homozygous wild type (C/C) melting temperature. Monitoring of the *C667T* genotype was visualized at 640 nm (by Light Cycler software version 4.0, Roche) and the melting curve showed a single peak at 62°C for *C/C* (homozygous wild type) samples, a single peak at 52.4°C for *T/T* (homozygous mutant types) samples, and two peaks for the heterozygous samples (consisting one *T/T* and one *C/C* allele) at 52.4°C and 62°C respectively. Positive control that was used consisted of lyophilised heterozygous DNA provided by manufacturers.

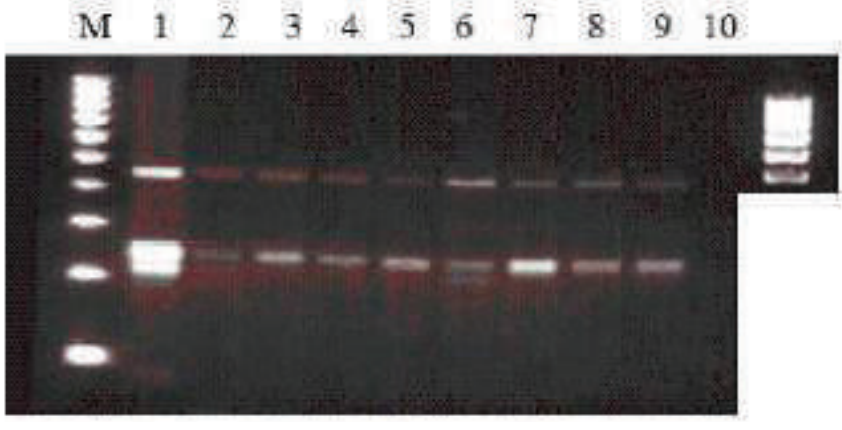

Figure 3. PCR analysis of TSER polymorphisms. Lane M shows a 100-bp DNA ladder. Lane 1, positive control (LS 174T cell line); most upper band (about 450 bp), a specific binding; Lane 2-9 patient samples; Lane 2 and 6 containing *2R/3R*; Lane 3,4,5,6,7,8,9 containing *3R/3R*. Lane 10, water control.

Data analysis and statistics

Frequencies of *TSER* polymorphisms between the Caucasian and Indonesian ALL samples and differences in clinical and hematological features were compared using the Chi-Square Test. One way anova was used for differences in age distribution and incidence of *TSER* polymorphisms. MTX IC50 was defined as the concentration causing 50% *TS* inhibition. Correlations between *TSER* repeats and IC50 were tested by Chi-Square Test. Statistics were performed on SPSS software, version 9.0 (Chicago, USA).

Results

Genotyping for *TS* tandem repeats

We analyzed the *TS* genotype in 101 samples of Indonesian (Javanese) 157 samples of Caucasian origin from leukemic cells using PCR assay and Genescan as described above. With gel electrophoreses we obtained PCR fragments with estimated lengths of 210 and 240 bp (Fig 3), which represent the two- and three-repeats sequences, respectively. From all samples extra PCR fragments longer than 240 bp were observed. These samples may contain *TS* repeats longer than three repeats or may be an aspecific binding of the primers. Because these fragments were not relevant to this study they were excluded from further analysis (fig 3). The difference in quantity of the two bands may be a result of Loss of Heterozygosity which phenomenon is also known in colorectal cancer (Bagley PJ and Selhub J, 1998; Kawakami K et al., 2002).

Table 1. TSER-P (polymorphisms)

TSER-P	n	Genotype			
		2R/2R	2R/3R	3R/3R	NA
Caucasian	157	32	72	52	0
		(20.4%)	(45.9%)	(33.1%)	
Indonesian	101	1	21	71	7
		(1.1%)	(22.6%)	(76.3%)	

TSER-P	MTX sensitivity		
	Sensitive	Intermediate	Resistant
3R/3R	13	8	4
	(52.0%)	(32.0%)	(16.0%)
*R/3R	13	41	2
	(23.0%)	(73.2%)	(3.6%)

A. Frequencies of TSER polymorphisms, between the Caucasian and Indonesian ALL population

B. MTX sensitivity associated with TSER genotype, in the Caucasian ALL population between the genotypes *R/3R and 3R/3R.

Data analyzed with Genescan showed PCR fragments of 208 and 236 bp probably depending on the Taq polymerase used for PCR. The frequency of each genotype in the 100 Indonesian samples and 157 Caucasian are shown in table 1. In contrast precisely, representing the two- and three-repeat sequences (fig. 5). The extra peak

in front of the main peak may be a result of hesitation of the tandem repeats, the frequency in *TSER* genotype was significantly different in Indonesian and Caucasian samples. The TS triple tandem repeat frequency was 33.1% in the Caucasian samples, which is corresponding with the 38% previously reported in Caucasians (Marsh et al., 1999). However, in the Indonesian samples, we observed a significantly higher triple repeat frequency of 76.3% (p< 0.001). The TS *2R/2R* genotype was present in 1,1% of the Indonesian samples and 20.4% in the Caucasian samples and the *2R/3R* repeat variant in 22.6% of the Indonesian samples and 45.9% in the Caucasians. In 7 cases the fluorescence intensity was to low for detection.

Genotyping of *C677T MTHFR* polymorphisms

The distribution of the *C677T* genotype was analyzed in 15 samples of Indonesian origin. The analysis of *MTHFR* genotype in Indonesia showed a frequency of 13.3% (n=2) for the heterozygous type *(C/T)* and 86.7% (n=13) was homozygote for *C667* (wild type). No complete homozygous mutation *(T/T)* in one these samples were observed (fig. 4). Because screening of this polymorphism was on such a small sample group no further analysis at moment was performed.

***TSER* polymorphisms and MTX sensitivity**

To determine whether there was a relation between *TS* polymorphisms and MTX sensitivity we analyzed the available data known from the Caucasian ALL samples. From the 157 samples, TS Inhibition Assay (TSIA) data was available from 81 samples. Inhibition of *TS* was determined in whole cells by measuring the *TS*-catalyzed conversion of 3H-dUMP to dTMP and 3H2O, as previously described (Rodenhuis S, et al., 1987; Rots MG et al., 1999). Briefly, 0.1x 106 cells were incubated in 150 αl culture medium with/without MTX (generously provided by Pharmachemie, Haarlem, The Netherlands). After 4 hours, [5-3H]-2'-deoxycytidine (final concentration 1 αM, 2.5 Ci/mmol) was added as precursor for 3H-dUMP. Data are expressed as TSI50 values, representing the concentration of MTX necessary to inhibit 50% of the TS activity either after a) short MTX exposure of 3 hours followed by an 18 hours drug free period (TSI50, short) or b) continuous MTX exposure for 21 hours (TSI50,cont.) (Rots MG et al., 1999).

Continuous long-term exposure to MTX

In this study, the data from continuous long-term exposure to MTX was used to determine MTX sensitivity. This data provided insight of only the polyglutamylated-

MTX and thereby the TS inhibition. Because the sensitivity for MTX below 0.156 αM and above 40 αM concentrations was undetectable, the measurements were divided into 3 categories; hypersensitive, intermediate and insensitive. To determine the effect of a triple repeat on MTX sensitivity the samples were divided in a group consisting at least one double repeat and a group with a triple repeat only (Table 1). In addition, a difference was found between the MTX sensitivity and the presence of a double or a triple repeats in Caucasian ALL population. Concerning the triple repeat there seems to be a shift in the distribution to hypersensitivity to MTX. However, four of the samples (16.0%) did show resistance to MTX. This could suggest an additional role for other enzymes involved in the folate metabolism, such as *MTHFR*.

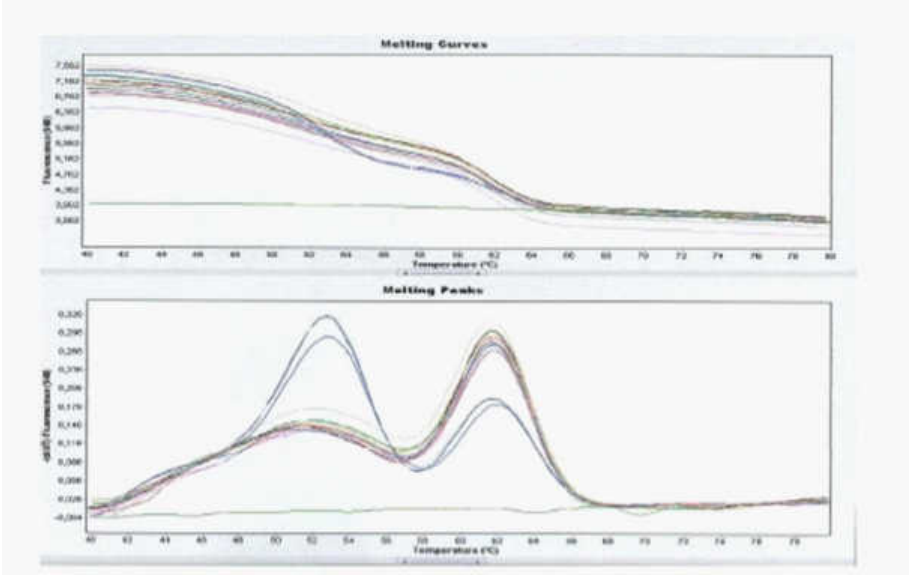

Figure 4. Typical example of melting curves and melting peaks used to genotype *C677T MTHFR* gene mutation. The probe and positive control that was used provided by the manufactures. Melting peak temperatures obtained from a derivative of the melting curves are 62.0 °C for homozygous wt (*C/C*), 52.4°C and 62.0°C for heterozygous (*C/T*). No homozygous mutant type (*T/T*) was observed.

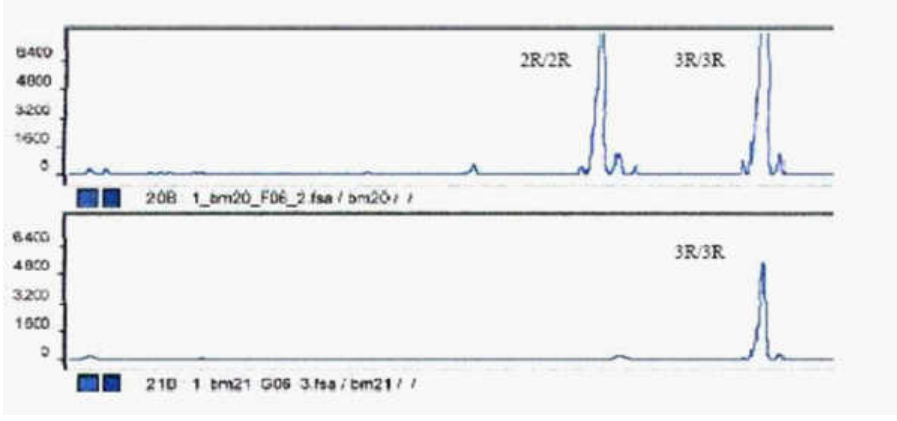

Figure 5. Genescan analysis of TSER polymorphisms. The PCR fragments with FAM-labelled reverse primer, was visualized with a DNA sequencer. (A) Represents a sample with a heterozygous genotype, consisting a double and a triple repeat. (B) Representing a sample with only a triple repeat

Discussion

Acute lymphoblastic leukemia is the most frequent malignancy that affects children. The key drug in the treatment of this leukemia is MTX, which by synthesis of MTX-polyglutamates acts as an inhibitor of *TS* (Rots MG et al., 2000). Ethnic variations of the polymorphic tandem repeat sequences in the enhancer region of the TS promoter (Marsh, 2004), has previously been described to influence the outcome of ALL (Krajinovic M et al., 2002). Mutations in the *MTHFR* gene, which regulates the intracellular folate homeostasis may also have an effect on the response of cancer cells to the chemotherapeutic agent MTX. Since polymorphisms can define the sensitivity of a therapy, identification of polymorphisms among different populations can be very important. This strengthens the need to examine the impact of cancer- treatment- related gene polymorphisms.

In this study, we compare the ethnic variations of polymorphisms in *TS* and *MTHFR* genotype in ALL cells, obtained at diagnosis from 157 Caucasian and 101 Indonesian children, which were about to be treated with MTX. Furthermore,we determined the involvement of TS polymorphisms in MTX sensitivity, by using a TS inhibition assay. In the present study, we found that the homozygous triple repeats were more than twice as common in Indonesian ALL samples (76.3%) than in Caucasian ALL samples (33.1 %). The Caucasian ALL data were completely corresponding with

the genotype frequencies previous reported in normal cells of healthy Caucasians (Marsh et al., 1999; Marsh S et al., 2000). This suggests that the genotype found in the Indonesian ALL samples may also represent their normal cells. However, to substantiate this hypothesis we will include normal cells of an Indonesian cohort in future studies. When the normal cells show a different distribution, it may be indicative of a different etiology of ALL in Indonesia. In any way these ALL data in the current study could be of great relevance in the treatment of ALL since the triple repeat may contribute to increased MTX sensitivity.

The difference in genotype was not associated with clinical and hematological features of the patient. There was no difference in sex and age distribution between patients consisting at least one double repeat or consisting a triple repeat only. Also no relation was found in WBC and FAB subtype distribution between the polymorphisms. According to several studies on TS polymorphisms, the triple tandem repeat is associated with gene expression and prognosis but giving rather contrasting results. An in vitro study has shown a stepwise increase in TS gene expression with increasing number of tandem repeats: the presence of a triple repeat results in 2.6 fold greater TS expression than a double repeat (Horie N., 1995). A retrospective study reported that triple repeat homozygous exhibit 3.6 fold higher TS mRNA levels as compared to double repeat homozygous (Pullarkat ST et al., 2001). Kawakami et al suggested that not the mRNA expression levels but the mRNA translation efficiency is responsible for the genotype dependent differences in *TS* expression (Kawakami K, et al., 1999). Etienne et al (2002) first described that there was no link between *TS* activity and *TS* polymorphisms (Etienne MC et al., 2002) but recent data (Etienne MC et al., 2004) showed that *TS* activity was significantly higher in *2R/3R* heterozygous human cell lines (head and neck, breast, digestive tract).

One of the major findings of the present study is that *TS* enzymatic activity is significantly influenced by the 5'TS genotype. Interestingly, according the data in the present study performed in Caucasian ALL samples, the triple repeat seems to be associated with hypersensitivity to MTX, which was unexpected data. This suggests a good treatment response and thereby a good prognosis. This in contrast with the results of Kraijnovic et al (2002). They reported a relation between *TS* polymorphisms and the prognosis of the patient. In 205 childhoods ALL cases homozygosity for the *TS* triple repeat was reported to be associated with poorer outcome than in those with at least one double-repeat (Krajinovic M et al., 2002). Similar findings have been reported for patients with colon cancer who received chemotherapy (Lacopetta,B, et al., 2001). Such contrasting results could be explained by other interacting enzymes or gene-gene interactions involved in

folate metabolism or an additional G → C SNP within the second repeat of the triple tandem may influence the transcriptional activity of the gene (Mandola MV et al.,2003). An additional polymorphism in the 5'regulatory region with functional consequences on transcriptional activity may explain the links between *TS* activity, tandem repeat polymorphisms and the prognosis of the patient. Four of the samples (16 %) with a triple repeat did show resistance to MTX, which may suggest an additional role for other enzymes. For instance *MTHFR*, a key enzyme of the folate metabolic pathway, and is subject to several polymorphisms (Rozen R, 1996). Among them the polymorphism at position *C677T* that exhibit significantly lower enzymatic activity, and should theoretically lead to an accumulation of intracellular 5,10-methylene THF concentrations as compared to WT forms. Accumulations of the 5,10-methylene THF may influence the intracellular concentration of folates, which is required for optimal *TS* inhibition. Recent clinical studies have suggested that *MTHFR* polymorphisms may be associated with MTX sensitivity (Ulrich CM et al., 2001; Urano W et al., 2002).

Kyoung-Jin et al, 2004, reported in the mutated *MTHFR* compared with a WT *MTHFR*, a decreased *MTHFR* activity, *MTHFR* thermolability, changed intracellular folate distribution, accelerated cellular growth rate, and increased *TS* activity. The *MTHFR 677T* mutation increased chemosensitivity with treatment of 5-fluorouracil (5-FU) but a decrease in chemosensitivity was seen in the treatment of MTX (Sohn KJ et al., 2004). Several studies reported that *MTHFR 677T* and *3R/3R TS* variants were found to influence susceptibility to ALL (Skibola CF et al., 2002; Skibola CF et al., 1999; Wiemels JL et al., 2001). Higher 5,10-methylene*THF* and *TS* levels associated with these variants were suggested to limit DNA damage by reducing uracil incorporation into DNA, thus explaining the protective role of these variants against leukemia. Krajinovic M et al, 2004 showed that polymorphisms in the *MTHFR* in combination with polymorphisms of the triple repeat in the *TS* gene resulted in highly significant reduction of event free survival (EFS) (Krajinovic M et al., 2004). These studies provide evidence of the critical role played by the enzymes *TS* and *MTHFR* in the folate pathway and their possible gene-gene interactions in MTX sensitivity and thereby ALL outcome.

In this study heterozygotes of the *MTHFR* mutations were seen in 13,3 % of the screened Indonesian samples (n=15), which is lower than frequencies 52,6 % seen in Caucasians previously reported (Etienne MC et al.,2004). Since polymorphism studies require large population groups the latter result did not allow further analyses concerning enzyme interactions with *TS* to be performed For this purpose the *MTHFR* polymorphisms will also have to be determined in the complete Indonesian cohort (n=105). In conclusion, this study shows ethnic variations

in the regulatory element of the *TS* gene in pediatric patients diagnosed with ALL. Furthermore, within the Caucasian ALL samples containing a triple repeat, hypersensitivity to MTX was observed. Although previous studies show conflicting results they were all associated with more resistance to MTX. The current study shows the opposite. Mutations in the *TS* enhancer region with or without other enzymes involved may influence gene expression, gene transcription and prognosis of the patient. Therefore it is important that further investigation will unravel the underlying mechanisms of different polymorphisms within the folate pathway, as well as their possible gene-gene interactions and their role as potential predictors of MTX responsiveness and/or toxicity.

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